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(54) Title: A METHOD FOR THE IDENTIFICATION OF PROTEASE INHIBITORS

(57) Abstract

Methods for detecting protease inhibitors are disclosed. Also described are DNA constructs and host cells transformed with these constructs for use in the subject methods. The methods utilize a host cell which exhibits a negative phenotype dependent on the activity of a given protease. Thus, inhibition of the protease confers a selectable phenotype on the cell. The negative phenotype can be conferred by either protease-mediated activation or inactivation of a protein conferring a selectable phenotype. The inhibitor is detected by transforming host cells expressing the genes for the selectable phenotype and a given protease with random peptide sequences. Inhibitors so identified can be used either directly or indirectly in the treatment of protease-dependent disorders.

5   A METHOD FOR THE IDENTIFICATION OF PROTEASE INHIBITORS

Technical Field
            The instant invention relates generally to the
identification of protease inhibitors. More
10 particularly, the invention relates to methods of
identifying viral protease inhibitors which can in turn
be used to treat or prevent viral infection.

Background
15   Proteases are enzymes that cleave peptide
bonds, thereby altering proteins. Besides degrading
proteins, these enzymes play a regulatory role in a
variety of physiological processes. Proteases fall into
four general classes: serine, cysteine, aspartic acid,
20 and metalloproteases. These classes are distinguished
primarily by mechanism (Dunn, B.M., in Proteolytic
Enzymes, R.J. Geynon and J.S. Bond, eds., IRL Press,
Oxford, 1989, pp. 57-82). Serine and cysteine proteases
have almost identical two-step mechanisms with an acyl-
25 enzyme intermediate. Together they comprise the majority
of the known proteases. Aspartic and metallo-proteases
catalyze direct hydrolysis of the peptide bond.
            Many procaryotic and eucaryotic proteins are
synthesized as larger biologically inactive precursors
30 which become activated only when acted upon by
endoproteases (proteinases). These enzymes typically
recognize specific domains, usually less than ten amino
acids in length including the sissile bond, in exposed
35 loops of generally loose secondary structure (Keil, B.,

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in *Methods in Protein Sequence Analysis*, M. Elzinga, ed.,
Humana Press, Clifton, N.J., 1982, pp. 291-304).

Maturation proteases are responsible for both
intracellular and extracellular cleavage of protein
5   precursors and many of the proteolytically processed
proteins in turn play key roles in physiological
abnormalities which give rise to disease states (Andrews,
P.C., et al., *Experientia* (1987) 43:784-789; Reich, E.,
et al., *Cold Spring Harbor Symposium: Proteases and
10   Biological Control*, Cold Spring Harbor Laboratory, Cold
Spring Harbor, 1975). Proteins that undergo
intracellular proteolytic maturation include secreted
proteins, lysosomal enzymes, mitochondrial proteins and
membrane proteins. These proteins are highly diverse in
15   function, having endocrine, neurological, and immune
functions, as well as acting as growth factors and
antibiotics. Secreted proteins that undergo
extracellular proteolytic processing include the plasma
zymogens involved in blood clotting and the immune
20   complement system.

Maturation proteases which are indirectly
involved in human disease are generally distinguished by
their high degree of substrate specificity. However, a
host of digestive proteases of lesser specificity are
25   also known and are more directly involved in diseases
such as chronic inflammation and tumor metastasis. These
enzymes include elastases, collagenases, mast cell
proteases, and extracellular matrix-degrading metallo-
proteases, among others.

30   Proteases also play key roles in many
infectious diseases. An obligatory step in the
replication of many pathogenic plant and animal viruses
involves virus-determined proteolytic processing of the
35   primary viral gene products (Hellen, C.U.T., et al.,

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Plant viruses which
encode proteases for this purpose include the
potyviruses, comoviruses, nepoviruses, sobemoviruses, and
luteoviruses. These viruses cause economically important

5   diseases in all major monocot and dicot families.
Similarly equipped animal viruses include the
picornaviruses, retroviruses, alphaviruses, flaviviruses,
pestiviruses, coronaviruses, and adenoviruses. Diseases
caused by these viruses include foot-and-mouth disease,

10   AIDS, the common cold, hepatitis and polio.

For example, Zucchini Yellow Mosaic Virus
(ZYMV), a potyvirus, expresses its genome as a single 350
kDa polyprotein which is cleaved into at least seven
mature gene products by three distinct proteolytic

15   activities. Two of the proteases are virus-encoded
(Dougherty, W.G., and J.C. Carrington, *Ann. Rev.
Phytopathol.* (1988) 26:123-143; Carrington, J.C., et al.,
EMBO J. (1990) 9:1347-1353), including the potyviral 49
kDa protease. This protease is responsible for at least

20   five of the seven cleavages. This enzyme is a trypsin-
like cysteine protease which is structurally and
mechanistically representative of the largest class of
viral proteases, including those of the animal
picornaviruses (Dougherty, W.G., et al., *Virology* (1989)

25   172:302-310; Bazan, J.F., and R.J. Fletterick, *Proc.
Natl. Acad. Sci. USA* (1988) 85:7872-7876). This enzyme
is highly specific and appears to recognize a region
comprised of about seven amino acids surrounding the
sissile bond (Dougherty, W.G., and T.D. Parks, *Virology*

30   (1989) 172:145-155). Of the five sites cleaved by this
enzyme, the two flanking the protease appear to be
cleaved intramolecularly, while the remaining three
appear to be cleaved intermolecularly (Garcia, J.A., et

35   al., *J. Gen. Virol.* (1990) 71:2773-2779). Of the latter

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three, the site between the NIb protein and the coat
protein appears to be the most active.

Disclosure of the Invention

5   The invention herein is based on the discovery
of a unique method for detecting peptide protease
inhibitors. These inhibitors can be used directly or
indirectly in the treatment of protease-dependent
diseases. Alternatively, the inhibitors so identified
10   can be utilized as structural models for the rational
design of peptide-mimetics.

Accordingly, in one embodiment, the subject
invention is directed to a method for detecting a
protease inhibitor which comprises:

15   (a) providing a population of host cells
expressing a first nucleic acid sequence encoding a
protease and a second nucleic acid sequence encoding a
protein capable of conferring a selectable phenotype on
said host cells dependent on the activity of said
20   protease;

(b) providing a pool of nucleic acid
constructs wherein at least one of the constructs in the
pool comprises a nucleic acid sequence encoding an
inhibitor of the protease;

25   (c) transforming the host cells of (a) with
the nucleic acid constructs of (b); and

(d) growing the transformed host cells of (c)
under conditions that distinguish cells with the
selectable phenotype, thereby detecting the presence of
30   the protease inhibitor.

In another embodiment, the subject invention is
directed to a DNA construct comprising:

(a) a first DNA coding sequence for a protein
35   capable of conferring a selectable phenotype on a host

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cell transformed therewith, the selectable phenotype dependent on the activity of a protease; and

          (b)   control sequences that are operably linked to the first and second coding sequences whereby the

5   coding sequences can be transcribed and translated in a host cell, and at least one of the control sequences is heterologous to at least one of the coding sequences.

          In an alternate embodiment, the DNA construct further includes a second DNA coding sequence for the

10   protease of interest.

          In yet another embodiment, the subject invention is directed to host cells stably transformed with these DNA constructs.

          These and other embodiments of the subject

15   invention will readily occur to those of ordinary skill in the art in view of the disclosure herein..

Brief Description of the Figures

          Figure 1 depicts Protease Inhibitor Selection

20   System I, as applied to ZYMV protease.

          Figure 2 depicts representative examples of Protease Inhibitor Selection System II.

          Figure 3 shows the strategy of cDNA synthesis from ZYMV and cloning methods.

25   Figure 4 shows the nucleotide sequence of the ZYMV genome (SEQ ID NO:1).

          Figure 5A shows the organization of the primary translation products of pZPro6, pZPro7 and placZα-CP. Figure 5B depicts the results of immunoblot analysis of

30   SDS/PAGE separated proteins from _E. coli_ cells harboring these plasmids.

          Figure 6 depicts the derivation of the pZPro7, pZPro9, pZPro10, pZPro11 and pZPro12 constructs and the

35   organization of the primary translation products. The

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open boxes denote ZYMV 49 kDa protease (Pro) cleavage sites. Strep^R = streptomycin-resistant. Amp^R = ampicillin-resistant, i.e., transformed. Cfu = colony-forming units. NT = not tested.

Detailed Description of the Invention

The practice of the present invention will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, virology, recombinant DNA technology, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Sambrook, Fritsch & Maniatis, Molecular Cloning: A Laboratory Manual, Second Edition (1989); Maniatis, Fritsch & Sambrook, Molecular Cloning: A Laboratory Manual (1982); DNA Cloning, Vols. I and II (D.N. Glover ed. 1985); Oligonucleotide Synthesis (M.J. Gait ed. 1984); Nucleic Acid Hybridization (B.D. Hames & S.J. Higgins eds 1984); Animal Cell Culture (R.K. Freshney ed. 1986); Immobilized Cells and Enzymes (IRL press, 1986); B. Perbal, A Practical Guide to Molecular Cloning (1984); the series, Methods In Enzymology (S. Colowick and N. Kaplan eds., Academic Press, Inc.); and Handbook of Experimental Immunology, Vols. I-IV (D.M. Weir and C.C. Blackwell eds., 1986, Blackwell Scientific Publications).

All patents, patent applications, and publications mentioned herein, whether supra or infra, are hereby incorporated by reference in their entirety.

A. Definitions

In describing the present invention, the following terms will be employed, and are intended to be defined as indicated below.

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By "protease" is meant an enzyme that cleaves a peptide bond. The term includes both endopeptidases (also called proteinases) which are proteases that hydrolyze internal peptide bonds, and exopeptidases,

5   which are proteases that cleave either N- or C-terminal peptide bonds. Some proteases are highly specific, cleaving only between two particular amino acids within a particular protein. Other proteases are less specific, cleaving between more than one amino acid pair and/or

10   cleaving between an amino acid pair in more than one location in the same and/or different proteins. Exemplary proteases include maturation proteases responsible for both intracellular and extracellular cleavage of protein precursors, such as secreted

15   proteins, lysosomal enzymes, mitochondrial proteins, membrane proteins, plasma zymogens, digestive enzymes, elastases, collagenases, mast cell proteases, extracellular matrix-degrading metalloproteinases; plant viral proteases such as proteases from potyviruses,

20   comoviruses, nepoviruses, sobemoviruses, and luteoviruses; and animal viral proteases such as proteases from picornaviruses, retroviruses, alphaviruses, flaviviruses, pestiviruses, coronaviruses, and adenoviruses.

25   By "protease inhibitor" is meant a molecule capable of altering the activity of a protease such that the protease is unable to completely hydrolyze a peptide bond for which it is specific. Protease inhibitors can be peptides composed solely of genetically encodable

30   amino acids. "Protease inhibitor" also encompasses synthetic peptide derivatives such as peptide aldehydes and ketones, peptide boronic acids, peptide chloromethyl ketones, azapeptides, peptide hydroxamic acids, and

35   peptide thiols. "Protease inhibitor" also encompasses

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synthetic nonpeptide compounds such as diisopropylphosphofluoridate, sulfonylfluorides, phosphoramidon, and halomethylcoumarins. For a detailed discussion of protease inhibitors, see Proteinase
5   Inhibitors, A.J. Barrett and G. Salvesen, eds. (Elsevier, Amsterdam, 1986).

The terms "peptide" and "protein" are used in their broadest sense, i.e., any polymer of genetically encodable amino acids (dipeptide or greater) linked
10   through peptide bonds. Thus, the terms include oligopeptides, polypeptides, protein fragments, muteins, fusion proteins and the like.

A "host cell" is a cell which has been transformed, or is capable of transformation, by an
15   exogenous nucleotide sequence. As described more fully below, host cells for use in the present invention may be either procaryote or eucaryote, depending on the specific protease in question and the selection system desired. In general, bacterial cells (either gram-negative or
20   gram-positive) are the hosts of choice when the protease and its dependent phenotype can be expressed in active form in these cells. Eucaryotic cells can be used, however, in cases where either the protease or its dependent phenotype can be adequately expressed only in
25   such cells, such as cases in which certain types of transport, metabolism, or post-translational modification are required. Eucaryotic cells can also be used to select inhibitors of other types of biological activities which can be expressed only in such cells, such as animal
30   virus replication. One skilled in the art can readily determine an appropriate host cell for use in the present invention.

A "replicon" is any genetic element (e.g.,
35   plasmid, chromosome, virus) that functions as an

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autonomous unit of DNA replication; i.e., capable of replication under its own control.

A "vector" is a replicon, such as a plasmid, phage, or cosmid, to which another DNA segment may be at-
5   tached so as to bring about the replication of the at-
tached segment.

A "double-stranded DNA molecule" refers to the polymeric form of deoxyribonucleotides (bases adenine, guanine, thymine, or cytosine) in a double-stranded
10   helix, both relaxed and supercoiled. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, _inter alia_, in linear DNA molecules (e.g.,
15   restriction fragments), viruses, plasmids, and chromosomes. In discussing the structure of particular double-stranded DNA molecules, sequences may be described herein according to the normal convention of giving only the sequence in the 5' to 3' direction along the
20   nontranscribed strand of DNA (i.e., the strand having the sequence homologous to the mRNA).

A DNA "coding sequence" or a "nucleotide sequence encoding" a particular protein, is a DNA sequence which is transcribed and translated into a
25   polypeptide _in vivo_ when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. A coding sequence can include, but
30   is not limited to, procaryotic sequences, cDNA from eucaryotic mRNA, genomic DNA sequences from eucaryotic (e.g., mammalian) DNA, and even synthetic DNA sequences. A transcription termination sequence will usually be
35   located 3' to the coding sequence.

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A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present

5   invention, the promoter sequence is bound at the 3' terminus by the translation start codon (ATG) of a coding sequence and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above

10   background. Within the promoter sequence will be found a transcription initiation site (conveniently defined by mapping with nuclease S1), as well as protein binding domains (consensus sequences) responsible for the binding of RNA polymerase. Eucaryotic promoters will often, but

15   not always, contain "TATA" boxes and "CAT" boxes. Procaryotic promoters contain Shine-Dalgarno sequences in addition to the -10 and -35 consensus sequences.

DNA "control sequences" refers collectively to promoter sequences, ribosome binding sites,

20   polyadenylation signals, transcription termination sequences, upstream regulatory domains, enhancers, and the like, which collectively provide for the transcription and translation of a coding sequence in a host cell.

25   A coding sequence is "operably linked to" another coding sequence when RNA polymerase will transcribe the two coding sequences into mRNA, which is then translated into a chimeric polypeptide encoded by the two coding sequences. The coding sequences need not

30   be contiguous to one another so long as the transcribed sequence is ultimately processed to produce the desired chimeric protein.

A control sequence "directs the transcription" of a coding sequence in a cell when RNA polymerase will

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bind the promoter sequence and transcribe the coding
sequence into mRNA, which is then translated into the
polypeptide encoded by the coding sequence.

      A cell has been "transformed" by an exogenous
5   nucleotide sequence when the sequence has been introduced
inside the cell membrane. An exogenous nucleotide
sequence may or may not be integrated (covalently linked)
to chromosomal nucleic acid making up the genome of the
cell. In procaryotes and yeasts, for example, the
10 exogenous nucleotide sequence may be maintained on an
episomal element, such as a plasmid. With respect to
most other eucaryotic cells, a stably transformed cell is
one in which the exogenous nucleotide sequence has become
integrated into the chromosome so that it is inherited by
15 daughter cells through chromosome replication. This
stability is demonstrated by the ability of the
eucaryotic cell to establish cell lines or clones
comprised of a population of daughter cell containing the
exogenous sequence.

20   A "clone" is a population of cells derived from
a single cell or common ancestor by mitosis. A "cell
line" is a clone of a primary cell that is capable of
stable growth in vitro for many generations.

      A "heterologous" region of a DNA construct is
25 an identifiable segment of DNA within or attached to
another DNA molecule that is not found in association
with the other molecule in nature. Thus, when the
heterologous region encodes a bacterial gene, the gene
will usually be flanked by DNA that does not flank the
30 bacterial gene in the genome of the source bacteria.
Another example of the heterologous coding sequence is a
construct where the coding sequence itself is not found
in nature (e.g., synthetic sequences having codons
different from the native gene). Allelic variation or
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naturally occurring mutational events do not give rise to
a heterologous region of DNA, as used herein.

The term "treatment" as used herein refers to
either (i) the prevention of infection or reinfection
5   (prophylaxis), or (ii) the reduction or elimination of
symptoms of the disease of interest (therapy).

B. General Methods

Described herein is a system which can be used
10 to select effective inhibitors of proteases from large
pools of random peptide sequences. The method utilizes a
known protease which can be obtained through standard
techniques, i.e. direct isolation, synthesis or
recombinant technology. The nucleotide sequence of the
15 protease can be determined and used to transform a host
cell. The host cell is also transformed with a
nucleotide sequence encoding a protein that confers a
negative phenotype on the cell, such as sensitivity to a
given antibiotic, or inability to grow on a given carbon
20 source, which is dependent on the activity of the cloned
protease. Genes for the protease and the protein
conferring the dependent phenotype are contained on one
or more constructs which have been introduced into the
host cell. Thus, inhibition of the protease confers a
25 selectable phenotype on the cell (e.g., antibiotic
resistance, or the ability to grow on a given carbon
source). Once identified, the particular inhibitor can
be isolated, sequenced and further used as described
below.
30 The negative phenotype may be expressed by
either of two general mechanisms. In the first, a gene
conferring a dominant negative phenotype is expressed as
an inactive precursor protein which is activated by
35 protease-mediated cleavage at a site which has been

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engineered to resemble a natural substrate of the protease. In the second mechanism, a gene conferring a selectable phenotype is inactivated by protease-mediated cleavage at a similarly engineered site.

5   The above-described host cells can then be used to detect effective inhibitors of the protease from large pools of random peptides encoded on another plasmid. Cells transformed with variants of this plasmid which encode effective inhibitors are identified by selection

10   for the appropriate phenotype. This additional plasmid contains a gene which encodes a "carrier" protein in which all or part of an exposed domain has been randomized with respect to its amino acid sequence. Typically, the randomized domain may range from four to

15   fifteen amino acids in length. The length of the randomized amino acid sequence will depend on the specific application of the inhibitor and can be readily determined by one skilled in the art. For example, peptides intended for use for the design of peptide

20   mimetics will tend to have shorter sequences than peptides for use in peptide or gene therapy.

        Part or all of this sequence is randomized with respect to the twenty genetically-encodable amino acids. Thus, a fully randomized set of heptapeptide sequences

25   would contain more than 10^9 different peptides.

        Such random sequence "libraries" can be constructed by replacing the sequence encoding the exposed domain in the "carrier" protein gene with a set of synthetic oligodeoxynucleotides of random sequence. A

30   natural substrate of the protease in question can be conveniently used for the "carrier" protein. Alternatively, one of the many well-characterized natural protease inhibitors may be used (Proteinase Inhibitors,

35   A.J. Barrett and G. Salvesen, eds. (Elsevier, Amsterdam,

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1986), section C), in which the amino acid sequence of
the native binding domain has been randomized.
Structural constraints placed on the randomized sequence
by the flanking domains of the "carrier" may be minimized
5   by flanking the randomized sequence with short "spacers"
of polyproline or polyglycine which are highly flexible
(Creighton, T.E., Proteins: Structures and Molecular
Properties, W.H. Freeman, New York, 1984, ch. 5).
        Some of these "random" peptides will, by
10   chance, have structures which are capable of binding
tightly to the active site of the protease, thereby
preventing it from either activating or inactivating the
negative phenotype-conferring protein, depending on the
mechanism employed. This, in turn, will confer the
15   selectable phenotype on the host cell when transformed
with these constructs. The structures of effective
inhibitors can then be determined by sequencing the
appropriate regions of constructs recovered from such
phenotype-selected cells.
20   A representative example of the first mechanism
described above, i.e., wherein the activation of a
negative phenotype-conferring protein is inhibited
(hereinafter referred to as Protease Inhibitor Selection
System I) as applied to the ZYMV protease is illustrated
25   in Figure 1. A portion of the ZYMV polyprotein is
depicted which includes the protease, replicase (NIb),
and coat protein (see U.S. Patent Application Serial No.
07/560,130). The arrows indicate substrate sites at
which the protease cleaves the polyprotein. In this
30   selection system, either the replicase or coat protein is
replaced with the coding sequence for the protein
conferring the negative phenotype. An example of the
latter includes E. coli ribosomal protein S12, which
35   confers sensitivity to streptomycin on streptomycin-

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resistant hosts such as E. coli strain MC1009 (Post, L.E., and M. Nomura, J. Biol. Chem. (1980) 255:4660-4666). A transcription repressor protein may also be used such as the lactose, tryptophan, or phage lambda
5   repressors (Lewin, B., Genes IV, Cell Press, Cambridge, 1990, pp. 240-264). These act by repressing expression of antibiotic resistance genes in hosts in which these genes are transcribed from repressible promoters. In either case the negative phenotype is only displayed when
10   the negative phenotype protein is being actively cleaved out of the polyprotein by the protease.

     For other proteases it may be convenient to express the protease and the negative phenotype precursor from separate transcription units. The only requirements
15   are that the negative phenotype protein be linked to an extraneous domain by a peptide sequence which is a natural substrate for the protease in question, and that this precursor be inactive until cleaved by the protease.

     Figure 2 illustrates the second mechanism
20   wherein the negative phenotype is conferred by protease-mediated inactivation of a protein conferring a selectable phenotype (referred to herein as Protease Inhibitor Selection System II). Examples of such proteins include secreted or membrane proteins which
25   confer resistance to the antibiotics ampicillin, tetracycline, or kanamycin (Methods in Enzymology, vol. 43, Academic Press, New York, 1975), or which confer the ability to utilize carbon sources such as lactose or maltose (Bieker, K.L., and T.J. Silhavy, Trends in
30   Genetics (1990) 6:329-334). These proteins are normally expressed as precursors in which an amino-terminal signal sequence directs transport of the protein across the cell membrane or insertion of the protein into the cell
35   membrane, after which the signal sequence is

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proteolytically removed. In these constructs, the
protease substrate peptide sequence is inserted between
the signal sequence and the mature protein such that
cleavage by the protease renders the protein incapable of
5   membrane transport or insertion and thereby inactive.
Alternatively, the protease substrate sequence may be
inserted into a surface domain of the mature protein such
that cleavage by the protease renders the protein ?
inactive.

10   A special case of this selection system occurs
with proteases which are toxic when expressed in E. coli
by virtue of their fortuitous inactivation of one or more
host proteins which are required for growth (Baum, E.Z.,
et al., Proc. Natl. Acad. Sci. USA (1990) 87:5573-5577).
15   In such cases, the inactivated host protein(s) confer the
selectable phenotype in the presence of inhibitors of the
protease.

          The random peptide inhibitor gene library may
be delivered to the selector cells by any of several
20   methods, the choice of which will depend to some extent
on the size of the library. One skilled in the art can
readily determine an acceptable technique to use with a
given library. For example, chemical transformation with
purified plasmid (Sambrook, J., et al., Molecular
25   Cloning, Cold Spring Harbor Laboratory, 1989, pp. 1.76-
1.84) can be used for libraries of up to 10^8-10^9 members,
depending on the efficiency. Such a library can
accommodate a complete set of fully randomized
pentapeptides. High voltage electroporation with
30   purified plasmid (Dower, W.J., et al., Nucleic Acids Res.
(1988) 16:6127-6145) is useful for libraries of 10^{10}-10^{11}
members, nearly sufficient to accommodate a complete set
of fully randomized heptapeptides. For larger libraries,
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bacteriophage-derived vectors can be used for delivery by transduction.

For example, a plasmid vector can be converted to a cosmid vector (Sambrook, J., et al., Molecular
5 Cloning, Cold Spring Harbor Laboratory, 1989, ch. 3) simply by insertion of a cos site and an appropriate length of "stuffer" DNA. Concatenate ligation of the library to such a vector can be followed by efficient packaging into phage λ pseudovirions using commercially
10 available preparations. Efficient, large-scale transductions of the packaged cosmids into selector cells can then be accomplished by established methods. In a further refinement, concatemers of the inhibitor gene library can be used instead of "stuffer" DNA in the
15 cosmid to achieve the necessary size for packaging. This reduces, by an order of magnitude, the number of transformants that need to be screened to cover the entire library.

The stringency of selection by these systems
20 can be adjusted in a variety of ways. A number of transcriptional promoters and enhancers of varying strengths are available (Sambrook, J., et al., Molecular Cloning, Cold Spring Harbor Laboratory, 1989, ch. 17), which can be used with the protease, negative phenotype
25 precursor, and inhibitor genes to raise or lower the inhibitor strength required for selection. Inducible promoters can be used, such that their strengths may be titrated by adjusting the amount of inducer in the growth medium. For example, by having the inhibitor expressed
30 from an inducible promoter, the potency threshold of a pool of selected inhibitors is raised, and the size of the pool reduced, simply by reducing the amount of inducer present during selection. In addition, such
35 inhibitor inducibility can be used to counterselect

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stable false positives, such as revertants that have
muta_ed the protease gene, simply by replica plating onto
selective medium in the absence of inducer. Only the
revertants are able to grow.

5   Once detected, the protease inhibitor can be
isolated and chemically characterized, using known
techniques. These systems can be used to generate
inhibitor peptides for any protease which can be
expressed in active form in a suitable host and for which
10   substrate cleavage site sequences are known. In addition
to the proteases of many important plant and animal viral
pathogens, inhibitors of the proteases of other types of
microbial pathogens as well as cellular proteases which
have been implicated in such disorders as rheumatoid
15   arthritis, Alzheimer's disease, and tumor metastasis, can
also be identified.

C.   Use and Administration
           The instant invention can be used to identify
20   protease inhibitors which in turn are useful in the
treatment of protease-dependent diseases in both plants
and animals. The inhibitors can be used directly in
peptide therapy or can be encoded in a gene and used in
gene therapy. The identified inhibitors can also serve
25   as structural models for the rational design of peptide-
mimetics, that is, synthetic compounds that mimic the
protease-binding action of the identified protease
inhibitors to bring reactive groups into contact with the
protease active site. (See, e.g., Demuth, H.-U., J.
30   Enzyme Inhibition (1990) 3:249-278). The present
invention also has a more general application in the
construction and use of in vivo systems for the selection
of bioactive peptides from peptide libraries. For
35   example, systems may be designed for the selection of

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peptide inhibitors of hydrolytic enzymes other than
proteases. A number of such enzymes are known which can
hydrolyze natural or artificial substrates to produce one
or more compounds which are toxic to E. coli (for

5   example, see Hydrolytic Enzymes, A. Neuberger and K.
Brocklehurst, eds. Elsevier, Amsterdam, 1987). The
expression of such an enzyme in an appropriate host cell
allows the selection of peptide inhibitors of the enzyme
based on their ability to confer viability on the cells

10   in the presence of toxigenic substrates.
        In general, any phenotype of cultured
procaryotic or eucaryotic cells which can be altered by
the endogenous expression of appropriate peptides such
that cells expressing such peptides can be readily

15   distinguished and isolated from cells which either do not
express such peptides or which express peptides which do
not alter the phenotype, may provide the basis for
establishing an in vivo system for the selection of
bioactive peptides from peptide libraries. Among the

20   most tractable medically important phenotypes will be
those manifesting susceptibility to microbial
pathogenicity.
        For example, the endogenous expression of a
random peptide library as an exposed domain of a suitable

25   stable "carrier" protein in a population of cultured
mammalian cells of sufficient size to ensure that all or
most members of the library are represented in the
population may be used to select peptides which interfere
with the ability of microbial pathogens such as viruses

30   or bacteria or their toxins to inhibit cell growth. When
such cell populations are challenged by such pathogens or
toxins, only those cells expressing inhibitory peptides
will grow, allowing the active peptides to be identified

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by established methods. Such peptides can in turn be
used for the development of effective therapies.

For the treatment of plant pathogenesis, the
identified inhibitors can be used to create transgenic
5   plants. One commonly used method of gene transfer in
plants involves the insertion of the gene of interest
into the T-DNA region of a Ti or Ri plasmid derived from
A. tumefaciens or A. rhizogenes, respectively. Many
control sequences are known which when coupled to a
10   heterologous coding sequence and transformed into a host
organism show fidelity in gene expression with respect to
tissue/organ specificity of the original coding sequence.
See, e.g., Benfey, P.N., and Chua, N.H., Science (1989)
244:174-181. Suitable control sequences for use in these
15   plasmids include promoters for constitutive leaf-specific
expression of the desired gene in the various target
plants. Other useful control sequences include a
promoter and terminator from the nopaline synthase gene
(NOS). The NOS promoter and terminator are present in
20   the plasmid pARC2, available from the American Type
Culture Collection and designated ATCC 67238. If such a
system is used, the virulence (vir) gene from either the
Ti or Ri plasmid must also be present, either along with
the T-DNA portion, or via a binary system where the vir
25   gene is present on a separate vector. Such systems,
vectors for use therein, and methods of transforming
plant cells are described in U.S. Patent No. 4,658,082,
and Simpson, R.B., et al., Plant Mol. Biol. (1986) 6:403-
415, incorporated herein by reference in their entirety.
30   Once constructed, these plasmids can be placed
into A. rhizogenes or A. tumefaciens and these vectors
used to transform cells of plant species which are
ordinarily susceptible to the particular plant pathogen.
35   The selection of either A. tumefaciens or A. rhizogenes

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will depend on the plant being transformed thereby. In
general, A. tumefaciens is the preferred organism for
transformation. Most dicotyledons, some gymnosperms, and
a few monocotyledons (e.g., certain members of the
5   Liliales and Arales) are susceptible to infection with A.
tumefaciens. A. rhizogenes also has a wide host range,
embracing most dicots and some gymnosperms, which
includes members of the Leguminosae, Compositae and
Chenopodiaceae. Alternative techniques which have proven
10   to be effective in genetically transforming plants
include particle bombardment and electroporation. See,
e.g., Rhodes, C.A., et al., Science (1988) 240:204-207;
Shigekawa, K., and Dower, W.J., BioTechniques (1988)
6:742-751; Sanford, J.C., et al., Particulate Science and
15   Technology (1987) 5:27-37; and McCabe, D.E.,
BioTechnology (1988) 6:923-926.

      Once transformed, these cells can be used to
regenerate transgenic plants. For example, whole plants
can be infected with these vectors by wounding the plant
20   and then introducing the vector into the wound site. Any
part of the plant can be wounded, including leaves, stems
and roots. Alternatively, plant tissue, in the form of
an explant, such as cotyledonary tissue or leaf disks,
can be inoculated with these vectors and cultured under
25   conditions which promote plant regeneration. Roots or
shoots transformed by inoculation of plant tissue with A.
rhizogenes or A. tumefaciens, containing the desired
gene, can be used as a source of plant tissue to
regenerate transgenic plants, either via somatic
30   embryogenesis or organogenesis. Examples of such methods
for regenerating plant tissue are disclosed in Shahin,
E.A., Theor. Appl. Genet. (1985) 69:235-240; U.S. Patent
No. 4,658,082; and Simpson et al., supra.

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The inhibitors identified by the present method can also be used in gene therapy. For example, HIV-specific protease inhibitor genes, in which a natural mammalian protease inhibitor serves as carrier for the

5   HIV protease inhibitor domain, can be used in anti-AIDS gene therapy. Lymphocytes or bone marrow cells from the patient can be transformed with the protease inhibitor gene _in vitro_ and returned to the patient, where they establish an HIV-resistant subpopulation of lymphocytes

10   which can gradually restore cell-mediated immune function as the patient's untransformed lymphocytes are depleted by the virus.

Similarly, proteases active in blood, lymph, or cerebro-spinal fluid which are essential components of

15   disorders such as chronic inflammations, metastatic cancers, and certain viral infections, may be targeted by protease inhibitor gene therapy, in which the inhibitors are secreted by transgenic lymphocytes or other transgenic cell implants.

20   For therapeutic use in animals, the inhibitors identified by the present method can be altered by established methods to improve their pharmaco-kinetic properties. For example, the inhibitors may be administered linked to a carrier. For example, a

25   fragment may be conjugated with a macromolecular carrier. Suitable carriers are typically large, slowly metabolized macromolecules such as: proteins; polysaccharides, such as sepharose, agarose, cellulose, cellulose beads and the like; polymeric amino acids such as polyglutamic acid,

30   polylysine, and the like; amino acid copolymers; and in-active virus particles. Especially useful protein substrates are serum albumins, keyhole limpet hemocyanin, immunoglobulin molecules, thyroglobulin, ovalbumin, and

35   other proteins well known to those skilled in the art.

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The protein substrates may be used in their native form or their functional group content may be modified by, for example, succinylation of lysine residues or reaction with Cys-thiolactone. A sulfhydryl

5   group may also be incorporated into the carrier (or inhibitor) by, for example, reaction of amino functions with 2-iminothiolane or the N-hydroxysuccinimide ester of 3-(4-dithiopyridyl) propionate. Suitable carriers may also be modified to incorporate spacer arms (such as

10   hexamethylene diamine or other bifunctional molecules of similar size) for attachment of peptides. Methods of coupling peptides to proteins or cells are known to those of skill in the art.

It is also possible to administer the

15   inhibitors identified using the instant method alone, or mixed with a pharmaceutically acceptable vehicle or excipient. Typically, the compositions are prepared as injectables, either as liquid solutions or suspensions; solid forms suitable for solution in, or suspension in,

20   liquid vehicles prior to injection may also be prepared. The preparation may also be emulsified or the active ingredient encapsulated in liposome vehicles. The active immunogenic ingredient is often mixed with vehicles containing excipients which are pharmaceutically accept-

25   able and compatible with the active ingredient. Suitable vehicles are, for example, water, saline, dextrose, glycerol, ethanol, or the like, and combinations thereof. In addition, if desired, the vehicle may contain minor amounts of auxiliary substances such as wetting or

30   emulsifying agents, or pH buffering agents. Actual methods of preparing such dosage forms are known, or will be apparent, to those skilled in the art. See, e.g., Remington's Pharmaceutical Sciences, Mack Publishing

35   Company, Easton, Pennsylvania, 15th edition, 1975. The

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composition or formulation to be administered will, in
any event, contain a quantity of the inhibitor adequate
to achieve the desired effect in the individual being
treated.

5   Additional formulations which are suitable for
other modes of administration include suppositories and,
in some cases, aerosol, intranasal, oral formulations,
and sustained release formulations. For suppositories,
the vehicle composition will include traditional binders

10   and carriers, such as, polyalkylene glycols, or
triglycerides. Such suppositories may be formed from
mixtures containing the active ingredient in the range of
about 0.5% to about 10% (w/w), preferably about 1% to
about 2%. Oral vehicles include such normally employed

15   excipients as, for example, pharmaceutical grades of
mannitol, lactose, starch, magnesium, stearate, sodium
saccharin cellulose, magnesium carbonate, and the like.
These oral compositions may be taken in the form of
solutions, suspensions, tablets, pills, capsules,

20   sustained release formulations, or powders, and contain
from about 10% to about 95% of the active ingredient,
preferably about 25% to about 70%.

Intranasal formulations will usually include
vehicles that neither cause irritation to the nasal

25   mucosa nor significantly disturb ciliary function.
Diluents such as water, aqueous saline or other known
substances can be employed with the subject invention.
The nasal formulations may also contain preservatives
such as, but not limited to, chlorobutanol and

30   benzalkonium chloride. A surfactant may be present to
enhance absorption of the subject proteins by the nasal
mucosa.

Controlled or sustained release formulations

35   are made by incorporating the inhibitor into carriers or

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vehicles such as liposomes, nonresorbable impermeable
polymers such as ethylenevinyl acetate copolymers and
Hytrel® copolymers, swellable polymers such as hydrogels,
or resorbable polymers such as collagen and certain

5   polyacids or polyesters such as those used to make
resorbable sutures. The inhibitors can also be delivered
using implanted mini-pumps, well known in the art.

         Furthermore, the inhibitors (or complexes
thereof) may be formulated into pharmaceutical composi-
10   tions in either neutral or salt forms. Pharmaceutically
acceptable salts include the acid addition salts (formed
with the free amino groups of the active polypeptides)
and which are formed with inorganic acids such as, for
example, hydrochloric or phosphoric acids, or such
15   organic acids as acetic, oxalic, tartaric, mandelic, and
the like. Salts formed from free carboxyl groups may
also be derived from inorganic bases such as, for
example, sodium, potassium, ammonium, calcium, or ferric
hydroxides, and such organic bases as isopropylamine,
20   trimethylamine, 2-ethylamino ethanol, histidine,
procaine, and the like.

         To treat an animal subject, the inhibitor of
interest is administered parenterally, usually by
intramuscular injection in an appropriate vehicle. Other
25   modes of administration, however, such as subcutaneous,
intravenous injection and intranasal delivery, are also
acceptable. Injectable formulations will contain an ef-
fective amount of the active ingredient in a vehicle, the
exact amount being readily determined by one skilled in
30   the art. The active ingredient may typically range from
about 1% to about 95% (w/w) of the composition, or even
higher or lower if appropriate. The quantity to be
administered depends on the animal to be treated and the
35   particular inhibitor used. Effective dosages can be

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readily established by one of ordinary skill in the art
through routine trials establishing dose response curves.
The subject is treated by administration of the
particular inhibitor, in at least one dose. Moreover,
5   the subject may be administered as many doses as is
required to effectively treat the individual.

Below are examples of specific embodiments for
carrying out the present invention. The examples are of-
fered for illustrative purposes only, and are not
10   intended to limit the scope of the present invention in
any way.

EXAMPLES

15   ### Example 1
The genetic expression of active ZYMV 49 kDa protease in E. coli.

This example describes the construction and
expression in E. coli of a gene which encodes a portion
20   of the ZYMV polyprotein. The primary translation product
of this gene is a 140 kDa protein which includes the
49 kDa protease and flanking cleavage sites, a portion of
the nuclear inclusion 'b' protein (NIb, also referred to
as the replicase), including the NIb/coat protein
25   cleavage site, followed by the coat protein (CP).
Evidence is presented showing that the expression of this
gene in E. coli leads to an accumulation of mature CP as
a result of efficient cleavage at the NIb/CP cleavage
site by the 49 kDa protease.

30

cDNA Cloning and Sequencing of the ZYMV Genome

A California isolate of ZYMV was obtained from
Professor J.A. Dodds of the University of California at
35   Riverside. The virus was propagated by mechanical

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inoculation of the cotyledons of ten-day-old <u>Cucurbita</u>
<u>pepo</u> cv. early straightneck seedlings. Systemically
infected leaves were harvested 3-8 weeks after
inoculation and virus was purified therefrom essentially
5   as described by Lisa, V., et al., <u>Phytopathol</u> (1981)
<u>71</u>:667-672. The virus was quantified by absorbance at
260 nm using an extinction coefficient of 2.8 A_{260}/mg/ml.
        Viral genomic RNA was isolated from purified
virions by digestion with protease K in borate buffer (pH
10 9) containing 1% SDS and 4 mM EDTA for one hour at 37°C,
followed by phenol/chloroform extraction and ethanol
precipitation. The RNA was redispersed in water,
quantified by absorbance at 260 nm, and analyzed by
agarose gel electrophoresis in the presence of methyl
15 mercuric hydroxide.
        DNAs complementary to ZYMV RNA were synthesized
essentially according to Gubler, U., et al. <u>Gene</u> (1983)
<u>25</u>:263-269, as described in the technical manual for the
Riboclone cDNA Synthesis Kit (Promega Corp.). Figure 3
20 shows an outline of this procedure. The first strand was
synthesized using AMV reverse transcriptase and an
oligodeoxythymidylate primer. After second strand
synthesis EcoRI linkers were added, digested, and the
cDNAs were ligated into the EcoRI site of pBluescript
25 (Stratagene, Inc.). The ligation product was then used
to transform competent <u>E. coli</u> XL-1 Blue cells
(Stratagene) which were then plated in the presence of
<u>lac</u> inducer (IPTG) and substrate (X-gal) for color
selection of recombinants. Plasmid DNA was isolated from
30 colorless clones by the alkaline lysis miniprep method
(<u>Molecular Cloning, A Laboratory Manual</u>, 2d Ed., J.
Sambrook, E. Fritsch, and T. Maniatis, eds., Cold Spring
Harbor Press, New York, 1989) and insert sizes were
35

estimated after digestion with EcoRI by agarose gel electrophoresis in the presence of ethidium bromide.

PZR1, the largest cDNA clone obtained from the first experiment, had a 2.3 kb insert, the ends of which
5   were sequenced using the Sanger dideoxy chain-terminating method as described in the product literature for the Sequenase 2 sequencing kit (United States Biologicals) with the M13 universal and reverse primers encoded on either end of the multiple cloning site in pBluescript.
10   The orientation of the insert relative to the viral genome and the multiple cloning site was indicated by the appearance of the polyadenylate tract from the 3' end of the genome in the sequence from the reverse primer. The remainder of the clone was sequenced stepwise, 200-300
15   nucleotides at a time, in both directions from synthetic oligodeoxynucleotide primers complementary to the distal ends of each of the successive sequencing runs. Sequence data were processed and analyzed on a DEC VAX 11/750 minicomputer.
20   The second round of cDNA cloning was accomplished in the same manner as the first except that a synthetic oligodeoxynucleotide complementary to the 5' end of pZR1 was used as primer and the cDNAs were ligated directly, without linkers, into the EcoRV site of
25   pBluescript (Figure 3). From this cloning two clones were obtained, pZB11 and pZB60, which had inserts of 2.3 kb and 3.8 kb, respectively. For the sequencing of pZB11, nested deletions were prepared from each end of the insert according to Henikoff, S., Gene (1984)
30   28:357ff as described in the product literature for the Erase-a-base System kit (Promega Corp.). For each direction, approximately twenty-four clones containing deletions spanning the entire length of the insert were
35   sequenced simultaneously from the M13 universal or

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reverse primers. Any gaps left by failure of the
sequences of adjacent time points to overlap were filled
in using synthetic oligodeoxynucleotide primers made from
the sequence near the 5' end of the gap.

5   Clone pZB60 was sequenced in both directions
from nested deletions in the same manner as for pZB11
except that a unique NcoI site within pZB11 was used as
the starting point for deletions in the 5' direction and
only those clones with deletions mapping between the 5'
10   end of pZB11 and the 5' end of pZB60 were sequenced.

A third round of cDNA cloning was conducted as
described above for the preparation of pZB11 and pZB60
except that a synthetic oligomer complementary to the 5'
end of pZB60 was used as a primer. From this round,
15   pZF18, having an insert of 3.7 kb was obtained and
sequenced as described above for pZB11 and pZB60.

The 5' end of the viral RNA sequence was
determined by reverse transcription of purified viral RNA
using a synthetic oligonucleotide primer complementary to
20   nucleotides 76-99 at the 5' end of pZF18. The Sanger
dideoxynucleotide chain-terminating method was used
essentially as described in the Promega Gem Seq manual
(Promega Corp.).

The continuous open reading frame of the viral
25   genome was identified with the aid of a computer as
described above. The coding sequences of the functional
ZYMV gene products were identified by amino acid sequence
homology to those of other potyviruses (Allison, R., et
al., Virology (1986) 154:9-20; Domier, L.L., et al.,
30   Nucleic Acids Res (1986) 14:5417-5430; Robaglia, C., et
al., J Gen Virol (1989) 70:935-947; Maiss, B., et al., J
Gen Virol (1989) 70:513-524). The identity of the coat
protein gene was further confirmed by subcloning the
35   presumptive coding sequence into a modified version of

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pBluescript from which the gene could be expressed _in_
vitro. In _vitro_ translation of the gene produced a
product of the expected size which reacted specifically
with antiserum raised against purified ZYMV coat protein
5   when analyzed by Western blotting.

Figure 4 shows the nucleotide sequence of the
ZYMV genome as determined above along with the deduced
amino acid sequence. The nucleotide sequence is numbered
from the 5' terminus. The 5' non-coding region extends
10   from nucleotide 1 to nucleotide 139. Nucleotides 140-142
initiate the polyprotein coding sequence with a
methionine codon in a consensus translation initiation
context (Joshi, C.P., _Nucleic Acids Res_ (1987) _15_:6643-
6653). By homology with the potyviral polyprotein
15   sequences cited above, the cleavage site between the
aphid transmission helper component (HC) and the 46 kDa
protein is believed to occur between the glycine at codon
766 (nucleotides 2435-2437) and the glycine at codon 767
(nucleotides 2438-2440). The cleavage site between the
20   46 kDa protein and the cytoplasmic inclusion protein (CI)
is believed to occur between the glutamine at codon 1164
(nucleotides 3629-3631) and the glycine at codon 1165
(nucleotides 3632-3634). The cleavage site between CI
and VPg/protease (VPg and protease are probably not
25   separated in ZYMV) is believed to occur between the
glutamine at codon 1798 (nucleotides 5531-5533) and the
serine at codon 1799 (nucleotides 5534-5536). The
cleavage site between VPg/protease and RNA replicase
(Rep) is believed to occur between the glutamine at codon
30   2284 (nucleotides 6989-6991) and the serine at codon 2285
(nucleotides 6992-6994). The cleavage site between the
RNA replicase and the coat protein (CP) is believed to
occur between the glutamine at codon 2801 (nucleotides
35   8540-8542) and the serine at codon 2802 (nucleotides

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8543-8545). Termination of the polyprotein coincides
with termination of the coat protein and is believed to
occur at the stop codon (nucleotides 9380-9382) following
the glutamine at codon 3080. The 3' non-coding sequence

5   then extends from nucleotide 9383 to nucleotide 9593
before terminating in a polyadenylate sequence of
variable length. cDNA clone pZR1 contained approximately
80 adenosines at its 3' terminus.

10   pZPro5

A restriction fragment of 1666 base pairs (bp)
extending between the PvuII and SspI sites of ZYMV cDNA
clone pZB11 (described above) was isolated by agarose gel
electrophoresis and ligated into the SmaI site of plasmid

15   pTZ18U (Sambrook, J., et al., Molecular Cloning, Cold
Spring Harbor Laboratory, 1989; Mead D.A., et al.,
Protein Engineering (1986) 1:67). This restriction
fragment comprises a portion of the coding sequence of
the ZYMV polyprotein which includes part of the

20   cytoplasmic inclusion protein (CI), the 6 kDa protein,
the 49 kDa protease, and a portion of the NIb protein.
Insertion of this fragment into the SmaI site of pTZ18U
places the reading frame encoding these proteins in phase
with that of the expressible lacZα gene of pTZ18U such

25   that expression of this gene from the lac promoter is
expected to produce a fusion protein comprised of a small
portion of the lacZα peptide fused to the amino terminus
of the ZYMV polyprotein fragment. This construct was
denoted pZPro5 and its structure was confirmed by

30   dideoxynucleotide sequencing (Sanger, F., et al., Proc.
Natl. Acad. Sci. USA (1977) 74:5463-5467).

pZPro6 and pZPro7

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The 2280 bp SalI restriction fragment from ZYMV cDNA clone pZR1 (described above), which comprises a portion of the ZYMV genome including part of the NIb protein, CP, the 3' non-coding sequence, and a portion of
5   the polyadenylate sequence, was inserted into the SalI site of pZPro5 to create pZPro6. Dideoxynucleotide sequencing of pZPro6 confirmed that the NIb/CP-encoding reading frame of the inserted fragment was in phase with the open reading frame of pZPro5 such that expression of
10   this construct from the lac promoter is expected to produce a single polypeptide of approximately 140 kDa.

In a further refinement of pZPro6, the 1244 bp MluI-NaeI fragment was removed and the 705 bp MluI-EcoRV fragment from pZR1 was inserted in its place, removing
15   most of the ZYMV 3' non-coding and polyadenylate sequences, which include several unwanted restriction sites. This construct was denoted pZPro7. E. coli strain DH5α was transformed with pZPro6 and pZPro7, and transformed clones were identified and isolated by
20   selection for ampicillin resistance.

Expression of the lacZα-ZYMV polyprotein gene in pZPro6 and pZPro7 was monitored by immunoblotting of SDS/PAGE-resolved proteins from these cells using polyclonal antisera raised in rabbits against denatured
25   ZYMV coat protein (Burnette, W.N., Anal. Biochem. (1981) 112:195). Results are shown in Figure 5B. Extract from cells harboring either pZPro6 or pZPro7 contained a single immunoreactive band which co-migrated with the major species of mature ZYMV coat protein at
30   approximately 31 kDa. Since the CP-containing primary translation product of 140 kDa was not detected in these extracts, the exclusive appearance of mature CP implies correct and efficient processing of the polyprotein by
35   the ZYMV 49 kDa protease.

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To rule out the possibility that mature CP might have been produced either by an endogenous _E. coli_ protease, or by fortuitous initiation of translation near the amino terminus of mature CP, a variant of pZPro7 was
5   also analyzed. This variant, denoted placZα-CP, was made by deleting the sequence encoding the protease and most of the NIb protein from pZPro7, leaving part of the NIb protein and CP in phase with the lacZα peptide in a 46 kDa open reading frame (see Figure 5A). Extracts from
10   cells harboring this construct contained a single immunoreactive band which migrated with an apparent MW of 46 kDa (Figure 5B, lane 2). The apparent absence in these cells of a species co-migrating with mature CP in the absence of the ZYMV 49 kDa protease indicates that
15   the activity of the latter is indeed responsible for the occurrence of mature CP in cells harboring pZPro6 and pZPro7.

Example 2
20   <u>Construction and analysis of genes which confer a negative phenotype on E. coli cells by virtue of the activity of the ZYMV 49 kDa protease according to the scheme described above for Protease Inhibitor Selection System I.</u>

25   This example describes the construction of expressible genes encoding polyproteins which contain the ZYMV 49 kDa protease and the _E. coli_ ribosomal protein S12. The ability of these gene constructs to confer sensitivity to the antibiotic streptomycin on several
30   streptomycin-resistant _E. coli_ strains by virtue of correct and efficient processing of the polyprotein by the ZYMV 49 kDa protease is demonstrated.

Streptomycin lethality in _E. coli_ has been
35   ascribed to its ability to interfere with protein

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synthesis by binding to the S12 subunit of the 30S
component of the ribosome (Gorini, L., in Ribosomes, M.
Nomura, A. Tissieres, P. Lengyel, eds., Cold Spring
Harbor Laboratory, 1974, pp. 791-803). Streptomycin-
5   resistant mutants have been isolated which express
altered forms of S12 which retain the ability to
participate in the assembly of ribosomes which can
function in the presence of streptomycin. Wildtype S12
has been shown to confer a dominant streptomycin-
10   sensitive phenotype on merodiploids which express both
wildtype and streptomycin-resistant forms of S12. The
highly sequestered position of S12 in the ribosome
suggests that S12-containing polyproteins should be too
encumbered to participate in the assembly of functional
15   ribosomes. Thus, the expression of such polyproteins in
streptomycin-resistant hosts should be unable to confer
streptomycin sensitivity unless mature S12 can be
proteolytically freed from the polyprotein.

20   pZPro9
The CP-encoding sequences in pZPro7 were
precisely replaced with the coding sequence for S12 to
create pZPro9. This was accomplished as follows. The
sequence bounded by the BglII site in NIb and the P1'
25   position of the NIb/CP cleavage site in pZPro7 (Schecter,
I., and A. Berger, Biochem. Biophys. Res. Commun. (1967)
27:157) was amplified by polymerase chain reaction (PCR,
Saiki, R.K., et al., Science (1988) 239:487). The S12
coding sequence from the second amino acid to the end was
30   amplified by PCR from plasmid pNO1523 (Dean, D., Gene
(1981) 15:99-102). The 3' primer contained an MluI site
following the stop codon. Following cleavage of the
first PCR product by BglII and the second by MluI both
35   were simultaneously ligated into pZPro7 from which the

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BglII-MluI fragment had been removed. Following
transformation and plasmid DNA purification, the
structure of pZPro9 (see Figure 6) was confirmed by
dideoxynucleotide sequencing.

5   pZPro9 was then transformed into streptomycin-
resistant E. coli strains MC1009, HB101, and N100
(American Type Culture Collection Catalogue of Bacteria
and Phages, 1989). After two rounds of single colony
isolation in the presence of ampicillin (amp), single

10   colonies of each transformant were grown in Luria-Bertani
medium (LB) containing 50 μg/ml amp to mid-log phase and
plated on solid LB containing 100 μg/ml amp, 100 μg/ml
streptomycin (strep), or both.
              Consistently, fewer than one in 10^5 amp-

15   resistant colony-forming units (cfu) of each transformant
was observed to grow in the presence of both amp and
strep, while the same hosts harboring pZPro7 plated with
similar efficiencies on amp alone or amp and strep (see
Figure 6). Thus, by virtue of having S12 in place of CP,

20   pZPro9 is able to confer strep sensitivity on strep-
resistant hosts, while its CP-containing parent, pZPro7,
is not. The pZPro9 transformants were fully sensitive to
as little as 3 μg strep/ml while the parent strains were
fully resistant to up to 350 μ/ml. Also, the pZPro9

25   transformants plated equally well on strep alone or amp
alone, indicating that pZPro9 is quickly lost in the
absence of amp selection and that there is no discernible
tendency to replace the strep-resistant gene in the host
chromosome with the S12 gene by homologous recombination.

30   To confirm that cleavage of the polyprotein by
the ZYMV 49 kDa protease to liberate mature S12 is
required for strep sensitivity, the NIb/CP cleavage site
was removed from pZPro9 to create pZPro12, which should
35   produce a polyprotein from which S12 cannot be freed by

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the protease. This was accomplished by cleaving pZPro9
with EcoRV and HpaI, which removed most of the NIb
protein including the NIb/CP cleavage site, and replacing
it with the fragment produced by EcoRV alone, which
5   restored most of the NIb protein down to within 12 amino
acids of the NIb/CP cleavage site (see Figure 6). The
structure of pZPro12 was confirmed by dideoxynucleotide
sequencing. Transformation with pZPro12 has no   ?
discernible effect on the ability of strep-resistant E.
10   coli strains to grow vigorously in the presence of up to
350 μg/ml streptomycin. Thus, the strep-sensitive
phenotype produced by pZPro9 is completely dependent on
the presence of a substrate cleavage site at which the
protease can cleave functional S12 from the polyprotein.

15   The expression of the pZPro9 polyprotein, like
most large eucaryotic proteins, places a considerable
burden on growing E. coli cells. In an attempt to reduce
this burden, the pZPro9 was streamlined by removing the
EcoRV fragment described above, which contains most of
20   the NIb protein exclusive of the cleavage sites at either
end. This construct, denoted pZPro10, encodes a
polyprotein of about 83 kDa, of which 49 kDa is the
protease and about 14 kDa is S12 (see Figure 6). Upon
transformation with pZPro10, strep-resistant E. coli
25   strains displayed a strep-sensitive phenotype identical
to that of the pZPro9 transformants. In addition,
pZPro10 transformants grew considerably more vigorously
than pZPro9 transformants, indicating a significant
reduction in the metabolic burden on the host cells.
30   Thus, removal of most of the NIb protein had no
discernible effect on the efficiency of removal of
functional S12 from the polyprotein by the protease.
             However, pZPro10-expressing cells still grew
35   poorly compared to the untransformed host. Recent work

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with another viral protease suggests that this is probably due, at least in part, to fortuitous activity of the protease on host proteins (Baum, E.Z., et al., *Proc. Natl. Acad. Sci. USA* (1990) 87:5573-5577). Inhibitors of
5 the protease should at least partly restore normal growth. As this growth differential is relatively easy to score, it is possible to use the toxicity of the protease as the negative phenotype, and to select inhibitors from peptide libraries, or to confirm selected
10 inhibitors on the basis of their ability to restore rapid growth.

Once the protease removes itself from the polyprotein of pZPro10, the 14 kDa S12 is left in a 21.5 kDa precursor until freed by the protease. To confirm
15 that neither this precursor nor the polyprotein itself is able to confer strep sensitivity, the NIb/CP cleavage site was removed from pZPro10 in the same manner that the EcoRV-HpaI restriction fragment was removed from pZPro9 to create pZPro12. This new construct, pZPro11, shown in
20 Figure 6, had no discernible effect on the level of strep resistance shown by strep-resistant *E. coli* strains. Thus, again, the presence of a substrate cleavage site adjacent to S12 is required for strep sensitivity, implying that the ZYMV 49 kDa protease is specifically
25 responsible for generating functional S12.

Thus, systems for identifying and selecting protease inhibitors from peptide libraries have been disclosed. Although preferred embodiments of the subject invention have been described in some detail, it is
30 understood that obvious variations can be made without departing from the spirit and the scope of the invention as defined by the appended claims.

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SEQUENCE LISTING

(1) GENERAL INFORMATION:

5

    (i) APPLICANT: BALINT, ROBERT

    (ii) TITLE OF INVENTION: A METHOD FOR THE IDENTIFICATION OF

10

        PROTEASE INHIBITORS

    (iii) NUMBER OF SEQUENCES: 1

    (iv) CORRESPONDENCE ADDRESS:

15

        (A) ADDRESSEE: Irell & Manella
        (B) STREET: 545 Middlefield Road, Suite 200
        (C) CITY: Menlo Park
        (D) STATE: CA
        (E) COUNTRY: USA

20

        (F) ZIP: 94025

    (v) COMPUTER READABLE FORM:

        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible

25

        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

    (vi) CURRENT APPLICATION DATA:

        (A) APPLICATION NUMBER: US 07/727,837

30

        (B) FILING DATE: 08-JUL-1991
        (C) CLASSIFICATION:

    (viii) ATTORNEY/AGENT INFORMATION:

35

        (A) NAME: ROBINS, ROBERTA L.

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                (B) REGISTRATION NUMBER: 33,208

                (C) REFERENCE/DOCKET NUMBER: 7115-0045

        (ix) TELECOMMUNICATION INFORMATION:

5   (A) TELEPHONE: (415) 327-7250

                (B) TELEFAX: (415) 327-2951

                (C) TELEX: 706141

10   (2) INFORMATION FOR SEQ ID NO:1:

        (i) SEQUENCE CHARACTERISTICS:

                (A) LENGTH: 9593 base pairs

                (B) TYPE: nucleic acid

15   (C) STRANDEDNESS: single

                (D) TOPOLOGY: linear

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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AAAGACTGAG CAGTGTGCAA ACACTCAAGT AAGTAATCGG GCTAATATAG TGGCACCTGG   240

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Claims

1. A method for detecting a protease inhibitor, said method comprising:

(a) providing a population of host cells expressing a first nucleic acid sequence encoding a protease and a second nucleic acid sequence encoding a protein capable of conferring a selectable phenotype on said host cells dependent on the activity of said protease;

(b) providing a pool of nucleic acid constructs wherein at least one of said constructs in said pool comprises a nucleic acid sequence encoding an inhibitor of said protease;

(c) transforming said host cells of (a) with said nucleic acid constructs of (b); and

(d) growing said transformed host cells of (c) under conditions that distinguish cells with said selectable phenotype, thereby detecting the presence of said protease inhibitor.

2. The method of claim 1 wherein said host cells are bacterial cells.

3. The method of claim 2 wherein said selectable phenotype is the ability of said bacterial cells to grow in the presence of a given antibiotic.

4. The method of claim 3 wherein said second nucleic acid sequence comprises a nucleic acid sequence encoding E. coli ribosomal protein S12 and said antibiotic is streptomycin.

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5.   The method of claim 2 wherein said first nucleic acid sequence comprises a nucleic acid sequence encoding ZYMV 49 kDa protease.

5   6.   The method of claim 2 wherein said selectable phenotype is the ability of said transformed host cells to grow in the presence of a given carbon source.

10   7.   The method of claim 1 wherein said second nucleic acid sequence comprises a nucleic acid sequence encoding a protein which is inactivated by said protease.

8.   The method of claim 1 wherein said second
15   nucleic acid sequence comprises a nucleic acid sequence encoding a protein which is activated by said protease.

9.   A DNA construct comprising:
(a)   a first DNA coding sequence for a protein
20   capable of conferring a selectable phenotype on a host cell transformed therewith, said selectable phenotype dependent on the activity of a protease; and
(b)   control sequences that are operably linked to said first and second coding sequences whereby said
25   coding sequences can be transcribed and translated in a host cell, and at least one of said control sequences is heterologous to at least one of said coding sequences.

10.   The DNA construct of claim 9 further
30   comprising a second DNA coding sequence for said protease.

11.   The DNA construct of claim 10 wherein said
35   protease is ZYMV 49 kDa protease.

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12. The DNA construct of claim 9 wherein said first DNA coding sequence codes for E. coli ribosomal protein S12 and said selectable phenotype is streptomycin resistance.

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13. The DNA construct of claim 10 wherein said first DNA coding sequence codes for E. coli ribosomal protein S12 and said selectable phenotype is streptomycin resistance.

10

14. The DNA construct of claim 11 wherein said first DNA coding sequence codes for E. coli ribosomal protein S12 and said selectable phenotype is streptomycin resistance.

15

15. A DNA construct comprising:

(a) a DNA coding sequence for a protein capable of inhibiting the action of a given protease, said protein identified by the method of claim 1; and

20

(b) control sequences that are operably linked to said coding sequence whereby said coding sequence can be transcribed and translated in a host cell, and at least one of said control sequences is heterologous to at least said coding sequence.

25

16. The DNA construct of claim 15 wherein said protease is ZYMV 49 kDa protease.

17. A host cell stably transformed with a DNA

30   construct according to claim 9.

18. A host cell stably transformed with a DNA construct according to claim 10.

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19. The host cell of claim 18 further
transformed with a DNA construct comprising:

(a) a DNA coding sequence for a protein
capable of inhibiting the action of a given protease,
5   said protein identified by a method comprising

(i) providing a population of host cells
expressing a first nucleic acid sequence encoding a
protease and a second nucleic acid sequence encoding a
protein capable of conferring a selectable phenotype on
10   said host cells dependent on the activity of said
protease;

(ii) providing a pool of nucleic acid
constructs wherein at least one of said constructs in
said pool comprises a nucleic acid sequence encoding an
15   inhibitor of said protease;

(iii) transforming said host cells of (i)
with said nucleic acid constructs of (ii); and

(iv) growing said transformed host cells
of (iii) under conditions that distinguish cells with
20   said selectable phenotype, thereby detecting the presence
of said protease inhibitor; and

(b) control sequences that are operably linked
to said coding sequence whereby said coding sequence can
be transcribed and translated in a host cell, and at
25   least one of said control sequences is heterologous to at
least said coding sequence.

20. A host cell stably transformed with a DNA
construct according to claim 11.

30

21. The host cell of claim 20 further
transformed with a DNA construct comprising:

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(a)   a DNA coding sequence for a protein capable of inhibiting the action of a given protease, said protein identified by a method comprising

(i)   providing a population of host cells

5   expressing a first nucleic acid sequence encoding a protease and a second nucleic acid sequence encoding a protein capable of conferring a selectable phenotype on said host cells dependent on the activity of said protease;

10   (ii)   providing a pool of nucleic acid constructs wherein at least one of said constructs in said pool comprises a nucleic acid sequence encoding an inhibitor of said protease;

(iii)   transforming said host cells of (i)

15   with said nucleic acid constructs of (ii); and

(iv)   growing said transformed host cells of (iii) under conditions that distinguish cells with said selectable phenotype, thereby detecting the presence of said protease inhibitor; and

20   (b)   control sequences that are operably linked to said coding sequence whereby said coding sequence can be transcribed and translated in a host cell, and at least one of said control sequences is heterologous to at least said coding sequence.

25

22.   A host cell stably transformed with a DNA construct according to claim 12.

23.   A host cell stably transformed with a DNA

30   construct according to claim 13.

24.   The host cell of claim 23 further transformed with a DNA construct comprising:

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(a)   a DNA coding sequence for a protein capable of inhibiting the action of a given protease, said protein identified by a method comprising

5
(i)   providing a population of host cells expressing a first nucleic acid sequence encoding a protease and a second nucleic acid sequence encoding a protein capable of conferring a selectable phenotype on said host cells dependent on the activity of said protease;

10
(ii)   providing a pool of nucleic acid constructs wherein at least one of said constructs in said pool comprises a nucleic acid sequence encoding an inhibitor of said protease;

(iii)   transforming said host cells of (i)

15   with said nucleic acid constructs of (ii); and

(iv)   growing said transformed host cells of (iii) under conditions that distinguish cells with said selectable phenotype, thereby detecting the presence of said protease inhibitor; and

20
(b)   control sequences that are operably linked to said coding sequence whereby said coding sequence can be transcribed and translated in a host cell, and at least one of said control sequences is heterologous to at least said coding sequence.

25

25.   A host cell stably transformed with a DNA construct according to claim 14.

26.   The host cell of claim 25 further

30   transformed with a DNA construct comprising:

(a)   a DNA coding sequence for a protein capable of inhibiting the action of ZYMV 49 kDa protease, said protein identified by a method comprising

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-54-

        (i)   providing a population of host cells expressing a first nucleic acid sequence encoding a protease and a second nucleic acid sequence encoding a protein capable of conferring a selectable phenotype on

5   said host cells dependent on the activity of said protease;

        (ii)   providing a pool of nucleic acid constructs wherein at least one of said constructs in said pool comprises a nucleic acid sequence encoding an

10   inhibitor of said protease;

        (iii)   transforming said host cells of (i) with said nucleic acid constructs of (ii); and

        (iv)   growing said transformed host cells of (iii) under conditions that distinguish cells with

15   said selectable phenotype, thereby detecting the presence of said protease inhibitor; and

        (b)   control sequences that are operably linked to said coding sequence whereby said coding sequence can be transcribed and translated in a host cell, and at

20   least one of said control sequences is heterologous to at least said coding sequence.
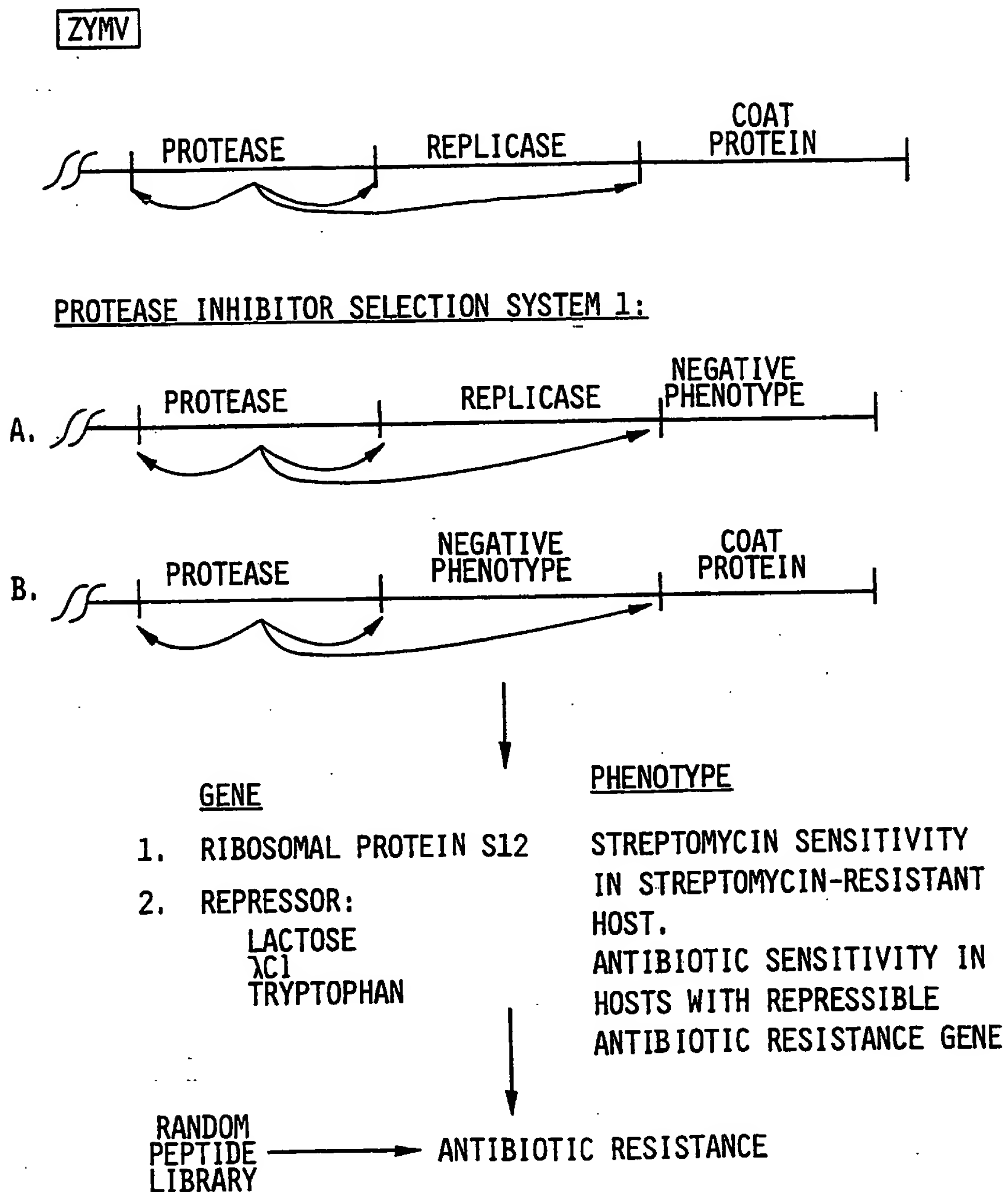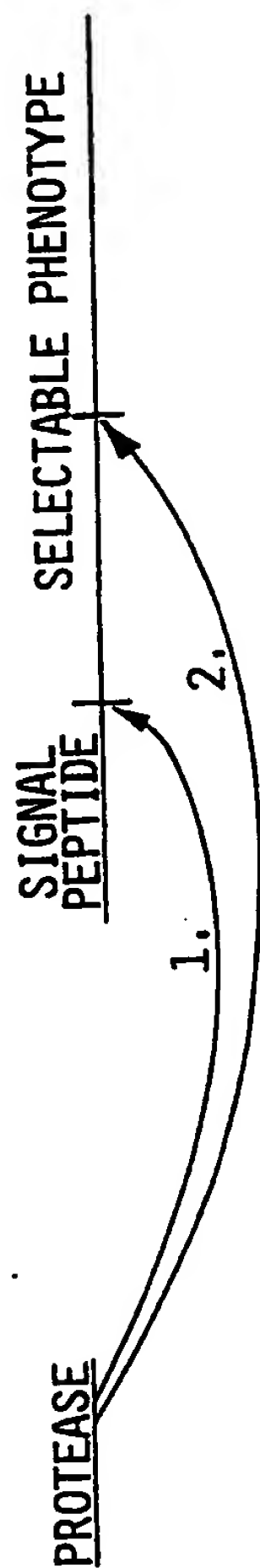
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ZYMV

| PROTEASE | REPLICASE | COAT PROTEIN |

PROTEASE INHIBITOR SELECTION SYSTEM 1:

A. | PROTEASE | REPLICASE | NEGATIVE PHENOTYPE |

B. | PROTEASE | NEGATIVE PHENOTYPE | COAT PROTEIN |

GENE

1. RIBOSOMAL PROTEIN S12

2. REPRESSOR:
   LACTOSE
   λCI
   TRYPTOPHAN

PHENOTYPE

STREPTOMYCIN SENSITIVITY IN STREPTOMYCIN-RESISTANT HOST.
ANTIBIOTIC SENSITIVITY IN HOSTS WITH REPRESSIBLE ANTIBIOTIC RESISTANCE GENE

RANDOM PEPTIDE LIBRARY ⟶ ANTIBIOTIC RESISTANCE

FIG. I

PROTEASE

SIGNAL
PEPTIDE

SELECTABLE PHENOTYPE

1. SECRETED OR MEMBRANE PROTEINS WITH CLEAVAGE SITE AFTER SIGNAL PEPTIDE.
   A. ANTIBIOTIC RESISTANCE-   B-LACTAMASE (AMPICILLIN)
                                 TETRACYCLINE
                                 NEOMYCIN PHOSPHOTRANSFERASE (KANAMYCIN)

   B. CARBON SOURCE UTILIZATION-LAC PERMEASE (LACTOSE)
                                 malF (MALTOSE)

2. CLEAVAGE SITE INSERTED INTO SURFACE DOMAIN OF ANTIBIOTIC RESISTANCE OR
   CARBON SOURCE UTILIZATION GENE.

FIG. 2
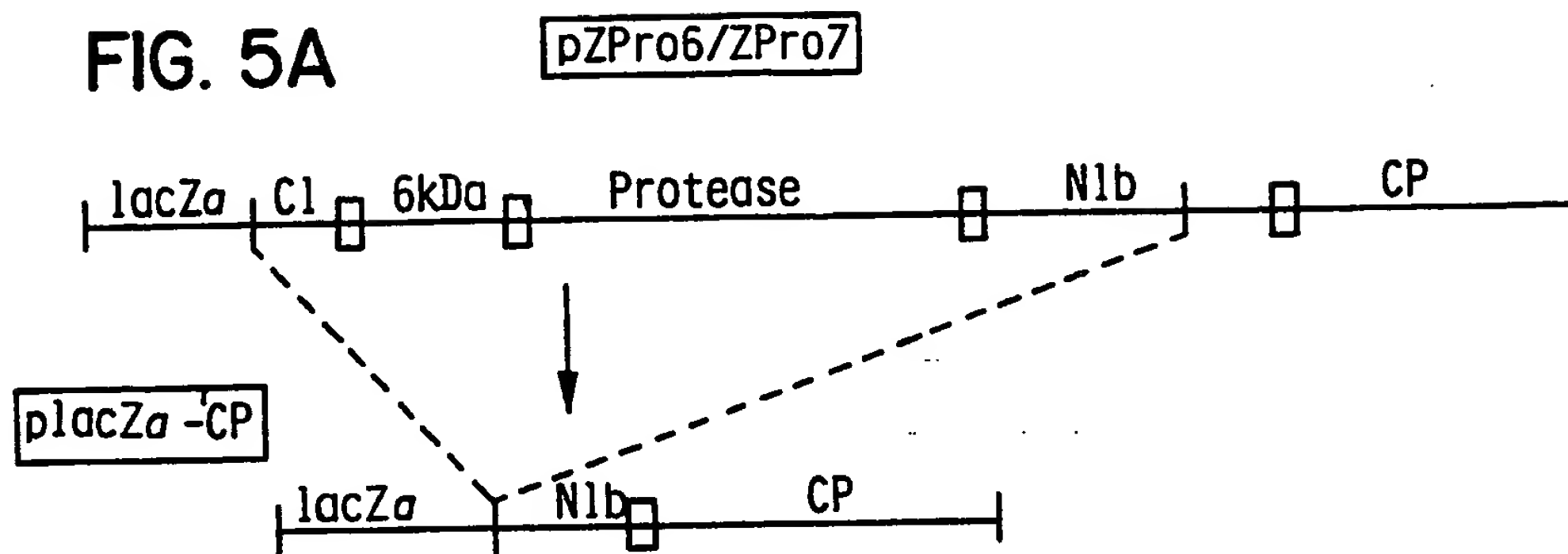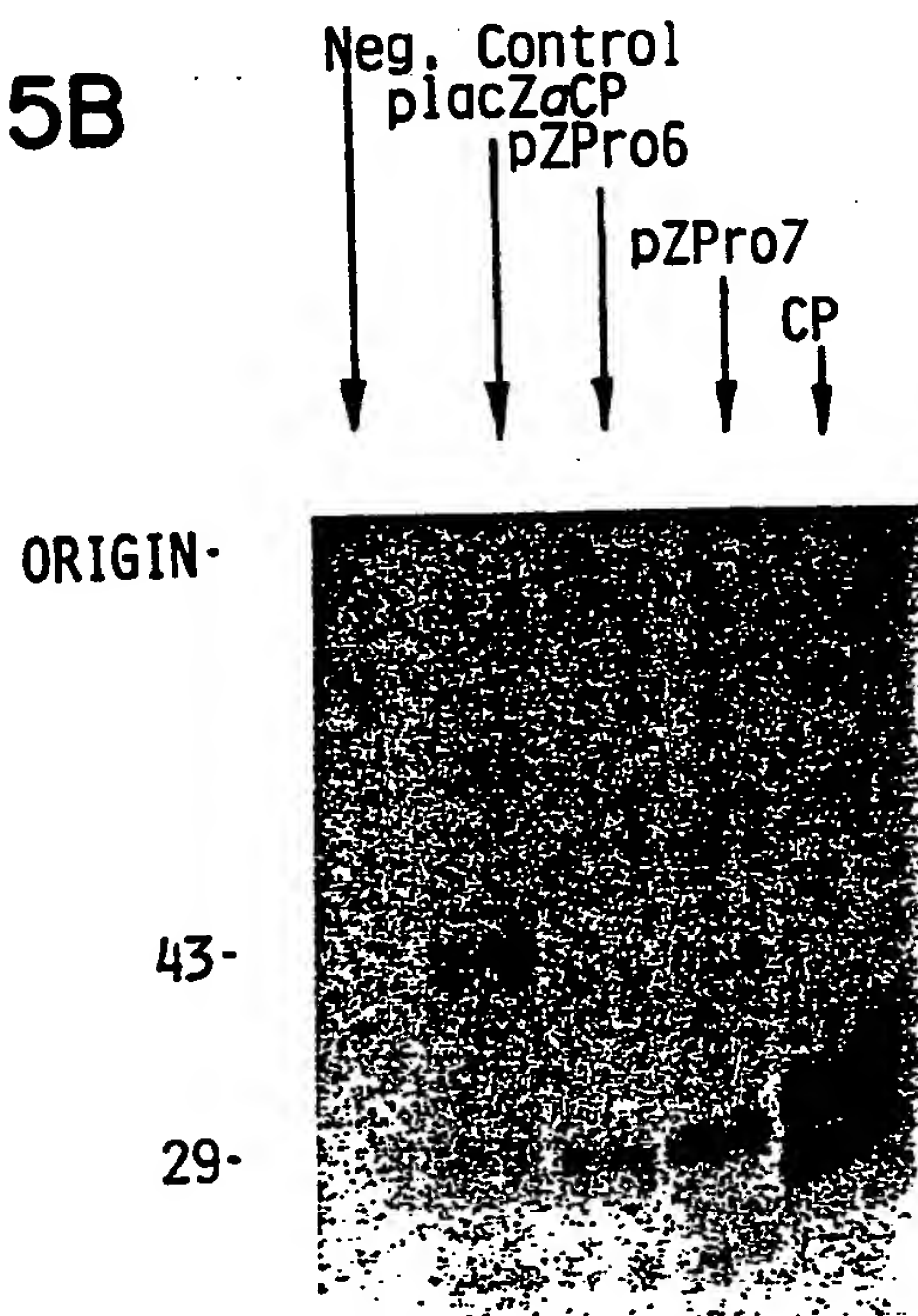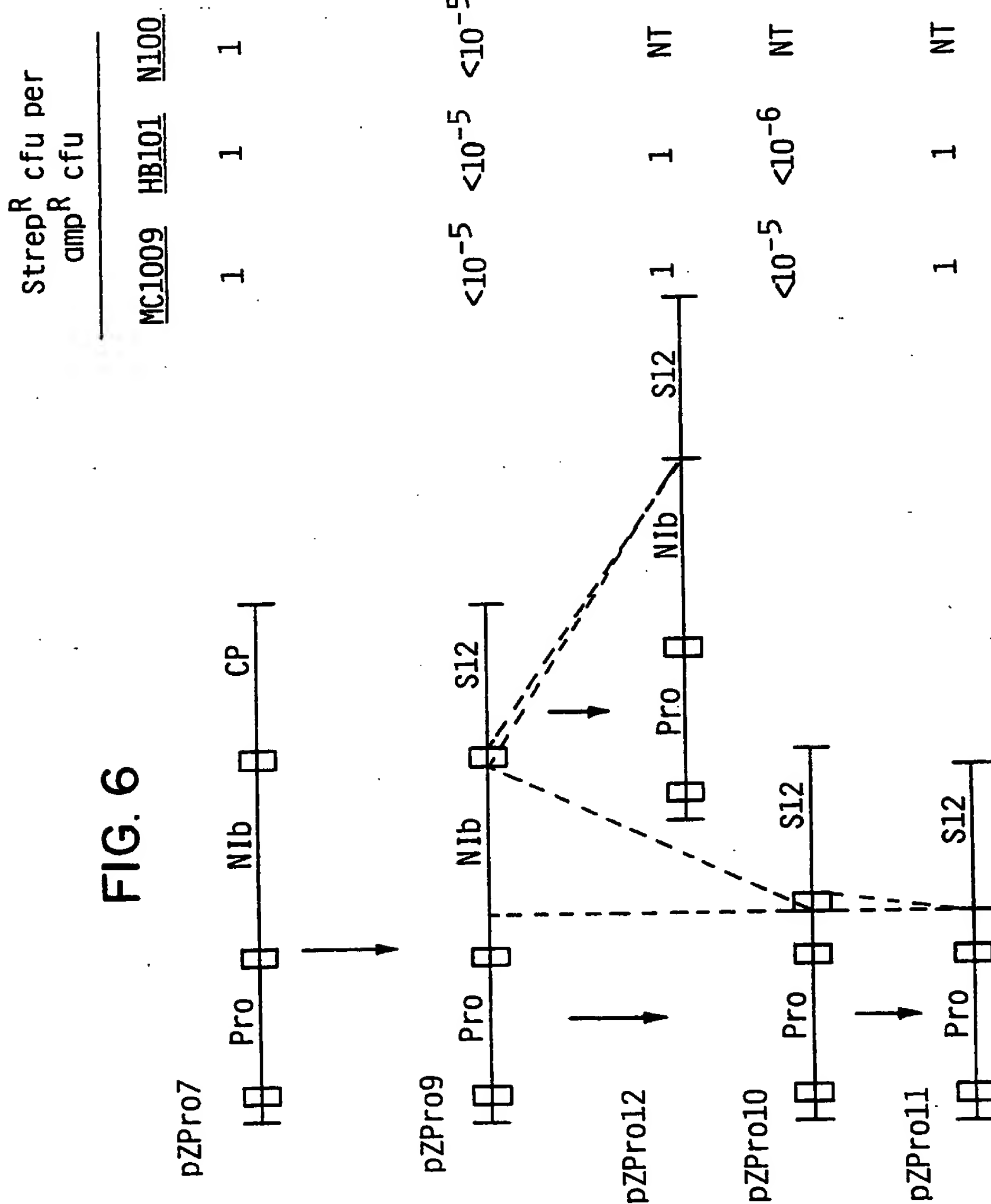
FIG. 3

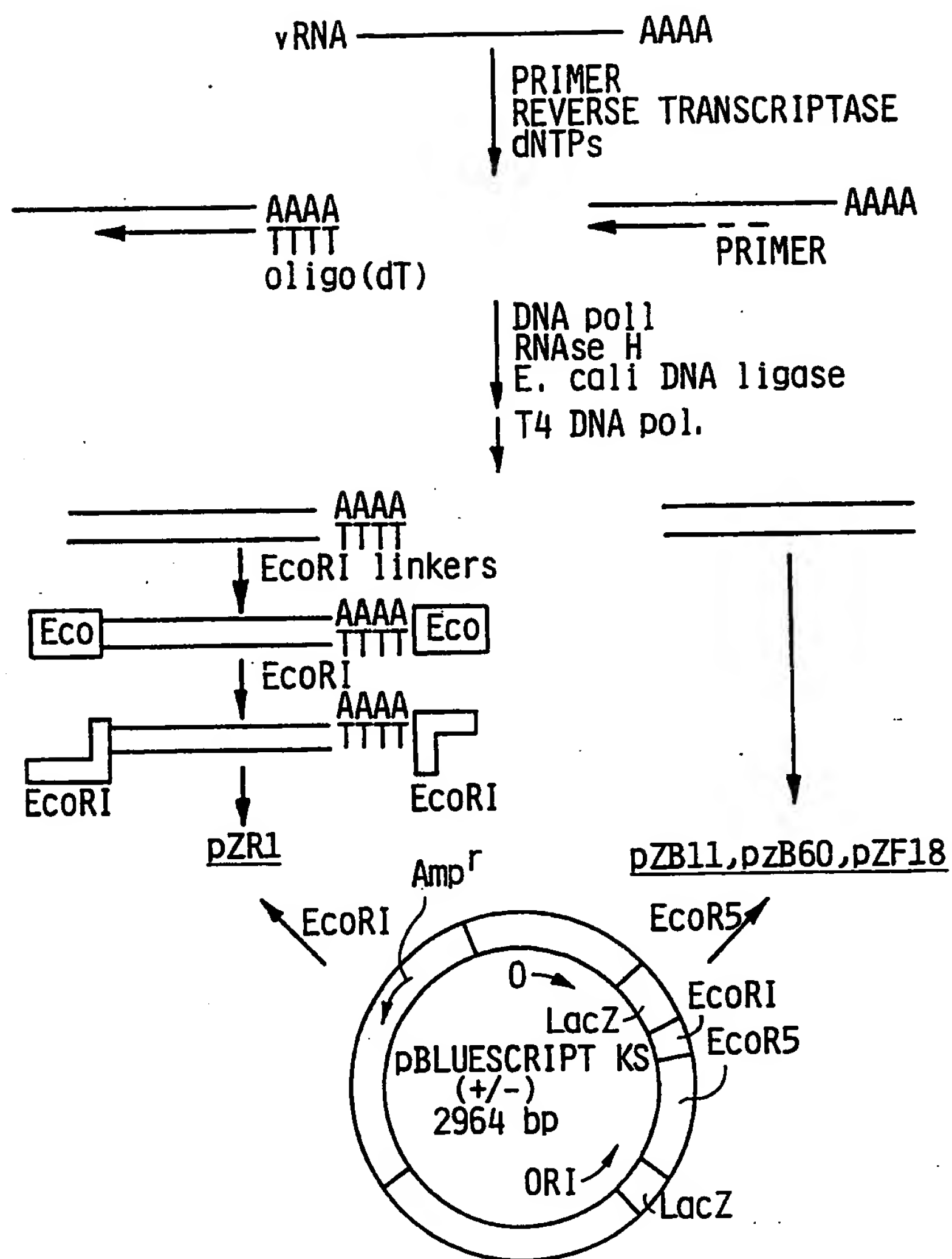# FIG. 5A

pZPro6/ZPro7

lacZa Cl 6kDa Protease Nlb CP

placZa -CP

lacZa Nlb CP

FIG. 5B

Neg. Control
placZaCP
pZPro6
pZPro7
CP

ORIGIN·

43·

29·

FIG. 6

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(5) :C12Q 1.02; C12N 15/15, 1.21
US CL :435/7.37, 320.1, 252.33; 536/27

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/7.37, 320.1, 252.33; 536/27

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, BIOSIS, WPI

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Proceedings of the National Academy of Sciences, Volume 87, issued December 1990, E. Z. Baum et al., "ß-Galactosidase containing a human immunodeficiency virus protease cleavage site is cleaved and inactivated by human immunodeficiency virus protease", pages 10023-10027, entire document.	1-26
Y	EP, A, 0,321,973 (Sommergruber et al.) 28 June 1989, entire document.	1-26
Y	Journal of General Virology, Volume 71, issued 1990, R. Grumet et al., "cDNA cloning and sequence analysis of the 3'-terminal region of zucchini yellow mosaic virus RNA", see especially Figure 1.	1-26
Y	Gene, Volume 15, issued 1981, D. Dean, "A plasmid cloning vector for the direct selection of strains carrying recombinant plasmids", see entire document.	1-26

☐ Further documents are listed in the continuation of Box C.   ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be part of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 August 1992	31 AUG 1992

| Name and mailing address of the ISA/ Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. NOT APPLICABLE | Authorized officer JAMES KETTER Telephone No.   (703) 308-0196 |

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